THE GENETICS OF DEET-RESISTANCE IN *CAENORHABDITIS ELEGANS*

by

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Bachelor of Science, 2007
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Submitted to the Graduate Faculty of the
College of Science and Engineering
Texas Christian University
in partial fulfillment of requirements
for the degree of

Master of Science

August 2009

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to my advisor, Dr. Phil Hartman, the committee members: Dr. Giridhar Akkaraju and Dr. Dean Williams, Dr. John Horner, and everyone in the biology department for their enlightening guidance and their helps with this project. I particularly thank Dr. Hartman for his patience and crucial support that have made this project possible.

I would like to dedicate this work to my mom and dad, who have empowered me, believed in me, and have always been with me through difficulties and successes. Their dreams and hopes have brought me up through the years. Their toils and tears have turned to the path for me to walk on.

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I. INTRODUCTION:

Olfaction affects organisms' perceptions of their surroundings and reality. Its role in species survival extends beyond just merely assisting organisms in looking for food. It is an intricate system that mediates the co-existence of the diversity, uniqueness, and originality among living organisms, such as speciation. Olfaction is controlled by olfactory receptor neurons (a.k.a. olfactory neurons, ORNs) that are stimulated by a diverse myriad of chemicals: organic and inorganic, volatile and non-volatile (Buck and Axel, 1991; Jacquin-Joly et al, 2004). These ORNs are highly conserved from invertebrate to vertebrate organisms, including mammals (Benton, 2006). In insects, olfaction inflicts different behaviors depending on the nature of the stimuli. Animals tend to chemotax towards attractants, avoid repellents, and not show any preference towards neutral chemicals. The perception of odorant, nevertheless, is arbitrary and may be influenced by other chemicals, such as DEET (Dogan, 1999).

N,N-diethyl-*m*-toluamide (DEET) is an active synthetic chemical in most topical insect repellent products. In 1998, data from the U.S. Environmental Protective Agency (U.S. EPA) showed that 225 DEET products were registered by 39 companies (30% of all insect repellent products), and that American consumers used 4 million pounds of DEET annually. Since its first development by the U.S. Army in 1946, DEET has been widely used and has benefited humans with the reduction in vector-borne diseases as well as the discomfort of insect bites caused by haematophagous arthropods. On the other hand, DEET has occasionally been related to adverse effects such as seizure, cardiovascular toxicity, or dermatitis, especially in children (U.S. EPA).

DEET is applied directly onto human skin or clothing and is available as an aerosol, cream, or lotion. Therefore, it is important to understand more about its action, starting with

how it affects invertebrate organisms. Despite its wide application as an insect repellent, the molecular mechanism of DEET action on insects has not successfully been elucidated. Early research on the action of DEET suggested that it inhibited the response of two classes of receptors on mosquito antennae that were sensitive to lactic acid, a chemical secreted by the human body that serves as an attractant to many species of mosquitoes and many other insects (Robbins & Cherniack, 1986). In the presence of DEET, a higher concentration of attraction was required to trigger the same level of response in the mosquito species *Aedes aegypt* (Dogan, 1999). Additionally, DEET has been shown to "inhibit" the attractive response in *Aedes aegypti* towards ethyl propionate, an oviposition attractant (Kuthilia, 1999). These data suggest that DEET is not a true "repellent." Rather, they suggest that DEET acts to inhibit olfaction. However, in 2001, Reeder *et al* (2001) isolated a Deet-Insensitive mutant of *Drosophila melanogaster* and, through several behavior-based experiments, proved that DEET was actually a repellent, though the gene that was responsible for the insensitivity phenotype has not yet been cloned.

The controversy over DEET action as a repellent is still unresolved. Two recent studies, both of which employed sophisticated electrophysiological measurements, have appeared in the literature. In March 2008, Ditzen *et al* (2008) reported in Science that DEET blocked electrophysiological responses of olfactory sensory neurons to attractive odors in *Anopheles gambiae* and *Drosophila melanogaster* and *Or83b* receptor was crucial for the response. *Or83b* is a ubiquitous co-receptor that was required for proper functions of olfactory sensory receptors in approximately 80% of antennal olfactory sensory neurons in insects. In *Drosophila*, they found that a 1,500-fold increase in the effective concentration of 1-octen-3-ol was required to trigger response in the neuron whose *Or83b* receptor is defective, and that the level of effect DEET imposed varied among neurons. The experiments

also confirmed the fact that DEET did not have a universal effect among all neurons and its strength differs between *Anopheles* and *Drosophila*. Virtually diametrically opposing these conclusions were those of Syed and Leal (2008), who concluded, "our research shows that mosquitoes smell DEET directly and avoid it." They performed single-unit recordings from all functional ORNs on the antenna and maxillary palps of the mosquito *Culex quinquefasciatus* and found that the same neuron that responds greatly to terpenoid compounds (i.e. thujone, eucalyptol, and linalool) also responded to DEET. Behavior tests on both male and female mosquitoes were also performed and showed no difference in behavior between the two sexes. Syed and Leal (2008) also found that there was not a decrease in response of the mosquito neuron towards 1-octen-3-ol when DEET is present. They suggested that the result from the Ditzen et al. (2008) study was a false positive and was an "experimental artifact." These experiments have reached different conclusions may be because DEET effect is species-dependent, or there may be a common molecular pathway by which DEET affects insects but proteins along the pathway determines the excitatory or inhibitory responses.

Caenorhabditis elegans is a good model organism to study DEET for many reasons: the *C. elegans* genome has been completely sequenced (The *C. elegans* Sequencing Consortium, 1998); *C. elegans* allows scientists to perform sophisticated molecular techniques such as RNAi (Ahringer, 2006); and most importantly , *C. elegans* behaves similarly towards DEET as many insects do. Wild-type *C. elegans* strain N2 normally senses and chemotactically responds to many volatile organic compounds (Bargmann et al., 1993).

Although *C. elegans* does not have visible antennae as insects do, this nematode does have quite sophisticated sensory neurons that help them detect a wide range of odorants (Bargmann et al., 1993). In addition, its nervous system has been developmentally well

described, thus giving researchers a firm foundation in attempting to elucidate chemosensory pathways. In *C. elegans*, the chemosensory neurons, consisting of the amphids, phasphids, and inner libial neurons (Ward et al., 1975; Ware et. al., 1975), respond to chemosensory cues, which results in a variety of behaviors. The cilia on these chemosensory neurons potentially interact with the environment through openings on the cuticle of the nematode or through rapid diffusion of a chemical through a thin sheath of cells. Additionally, many attractants and repellents to which *C. elegans* responds have been identified, which is an advantage in working with *C. elegans* as a model system.

Research in our laboratory has previously demonstrated that *C. elegans* was not repelled by DEET but that the incorporation of DEET into the test medium inhibited their ability to move towards attractants (Freedman, 2006). This is consistent with the findings by Dogan *et al* (1999); namely, that DEET by itself was the attractant of the mosquitoes *Aedes aegypti* yet became an inhibitor when DEET and another attractant were present together. The data by Freedman also suggested that DEET might interfere upstream of some neuronal circuits. From the preliminary data described above, a forward genetics approach was employed to search for a gene and the protein that is involved in DEET olfaction. Using the mutagen EMS, several mutants called DEET-resistant mutants (*der*) were isolated based on the premise that they were able to sense volatile organic attractants on a 0.002M DEET plate (Copeland, 2006). Single nucleotide polymorphism mapping (SNP) data have shown that the gene that is responsible for the DEET-resistance phenotype is on Linkage Group IV, and some precedent 3-Factor crosses suggested that the *der-1* is located between 1.78 (Unc-5) and 3.34 (Egl-19) Map Units (mu).

The location of *der-1* was first narrowed using 3-Factor crosses, and then deficiency mapping, candidate gene screening, and RNAi experiments were conducted with the goal of

cloning the *der-1* gene. Successful cloning of *der-1* may enable us to elucidate the molecular mechanism of DEET on olfaction to design the next generation of insect repellents. This thesis reports the location of *der-1* within a 400-kbp region on Chromosome IV and a discovery on DEET-resistance status among different wild-type strains of *C. elegans*.

II. MATERIALS AND METHODS:

Strains and genetics

The *C. elegans* used in this project were either purchased from the Caenorhabditis Genetics Center at the University of Minnesota in Minneapolis, Minnesota, or came from the laboratories of either Dr. Cornelia Bargmann of Rockefeller University, New York City, New York, or Dr. Shohei Mitani of Tokyo Women's Medical University, Tokyo, Japan. **Wild-type strains** (with their isolation location listed in parentheses) consisted of N2 (Bristol, England), AB1 (Adelaide, Australia), AB2 (Adelaide, Australia), CB4852 (Rothamsted, England), and CB4856 (Hawaii, U.S.A). **Mutant strains for mapping** included CB152 [*unc-5(e152) IV*], CB184 [*dpy-13(e184) IV*], CX2386 [*odr-8(ky31) IV*], CX2398 [*odr-8 (ky41) IV*], CX173 [*odr-8(ky173) IV*], CX2377 [*odr-8(ky28) IV*], CX2375 [*odr-8(ky26) IV*], CX5770 [*unc-44; kyls156 (str-1:odr-10 CDNA-GFP int. on X)*], MT2121 [*lin-33(n1043) IV*], CB1197 [*unc-44(e1197) IV*], PS427 [*lin-45(sy96) IV*], BE16 [*bli-6(sc16) IV*], EH135 [*unc-44(e362) bli-6(sc16) IV*], MT5240 [*unc-5(e53) lin-33(n1043) bli-6(sc16) IV*], and MT1212 [*egl-19(n582) IV*]. **Mutant candidates** included RB1804 [*T22D1.11(ok2338) IV*], VC8 [*jnk-1(gk7) IV*], RB1961 [*C06G3.7(ok2580) IV*], TM2701 [*srg-50 IV*], TM2717 [*srg-50 IV*], TM0418 [*C06G3.6 IV*], TM3367 [*nhr-249 IV*], TM1498 [*T22D1.12 IV*], [*srv-17* (ttTi27648)], and [*srv-22* (iiTi7473)]. **Deficiency strains** were BC1216 [*sDf21 dpy-4/nT1 IV; +/nT1 V*], BC1217 [*sDf22/nT1 IV; +/nT1 V*], CB3824 [*eDf19/unc-24(el38) dpy-20(el282) IV*], DA768 [*bli-6(sc16) egl-19(ad695) unc-24(el38)/nDf41 IV*], DR684 [*mDf9/nT1 IV; +/n T1 V*], DR814 [*dpy-13(e184) ama-1(m118)mDf8 IV/ nT1[let?(m435)] (IV, V)*], DR918 [*mDf10/nT1 IV; +/nT1 V*], DR799 [*mDf4/nT1; +/nT1 V*], and RW1350 [*unc-44(e362) unc-82(el323)/ stDf7*]. **Strains that were generated in this project** were PH250 [*der-1(hf175) IV*], PH251 [*der-1(hf176) IV*], PH252

[*unc-44(e1197) egl-19(n582) IV*], PH253 [*unc-5(e152) lin-33(n1043) IV*], PH254 [*unc-5(e152) egl-19(n582)*], PH255 [*der-1(ky176) kyls156 (str-1:odr-10 CDNA-GFP int. on X)*], and PH256 [*der-1(ky176)kyls156 (str-1:odr-10 cDNA-GFP int. on X)*]. **RNAi strains** consisted of T22D1.3(*IV-3K12*), IV-3K10(*IV-3K10*), B0478.2(*IV-3M10*), C25A8.2(*IV-3006*), C06G3.8(*IV-3006*), C06G3.8(*IV-3B01*), C06G3.9(*IV-3B03*), and C06G3.3(*IV-3016*). These strains were obtained through the courtesy of Dr. Rueyling Lin, University of Texas Southwestern Medical Center, Dallas, Texas.

Each *C. elegans* strain was maintained on an agar surface in 60x15mm petri dishes with either OP50 or DA837 *E. coli* under un-crowded conditions (Brenner, 1974). The streptomycin-resistant DA837 strain was acquired through the courtesy of Dr. Jim Waddle, Southern Methodist University, Dallas, Texas. All animals were maintained according to the method developed by Sydney Brenner (1974).

Assay Plate Preparation

Nematode Growth Medium (NGM) Plates were prepared by adding the following ingredients to 1 L of deionized (DI) water: 17g agar, 3g NaCl, and 2.5 g peptone. The solution was autoclaved and allowed to cool to 52^{o}C before adding: 1ml of 5mg/ml cholesterol in ethanol, 1ml of 1M $MgSO_4$, 25 ml of 1M $KHPO_4$ buffer pH 6.0, 1 ml of 1M $CaCl_2$, 2ml of 12mg/ml nystatin, and 0.1g streptomycin (final concentration 0.1mg/ml). Streptomycin was added only when DA837 was used. DA837 is a streptomycin-resistant strain that helps suppress bacterial contamination.

RNAi NGM plates were prepared followed the NGM recipe above with the addition of ampicillin (final concentration 50μg/ml) and IPTG (Isopropyl-Beta-d-Thiogalactopyranoside, final concentration 1mM) to the 52^{o}C post-autoclaved agar solution.

DEET Testing Plates followed the recipe of Bargmann et al. (1993) and were

prepared by adding 17g of agar and 25ml of 1M $KHPO_4$ buffer pH6.0 to 1L of DI H_2O. The solution was autoclaved and allowed to cool to approximate 60^oC before the addition of 2ml of 1M DEET solution in ethanol. It then was mixed and poured into 100x15mm Petri Dishes (approximately 25ml/plate). Plates were left to dry at room temperature (25^oC) for 48 hours before being stored at 4^oC for a maximum of 15 days.

Chemotaxis Testing Plates were made according to the DEET Testing Plate recipe without the addition of DEET .

Chemotaxis Assay

The **DEET Sensitivity Assay** was performed to test the chemotactic sensitivity of different *C. elegans* strains in the presence of DEET toward isoamyl alcohol (IAA), which was diluted in ethanol (Freedman, 2006). To prepare the animals for the test, adults were washed with deionized (DI) water into a glass test tube and allowed 3-5 minutes to settle to the bottom of the tube. The excess water was then aspirated to approximately 0.5 ml. The wash was repeated three times to make sure that most bacteria being transferred from the NGM plates were diluted such that they no longer influenced olfaction. Meanwhile, a line was drawn on the back of the DEET plate to divide it in half. At the middle axis of one end, a circle was marked that was approximately one centimeter in diameter. This is where the IAA was placed. After the washing stage, 15µl of the water containing ~100-200 animals was transferred on to one end of an agar DEET plate. A platinum wire was used to spread the animals horizontally. 2.5µl of 0.1M IAA was added at the circle every 15 minutes. The total worm population on the plate was assessed. After each 15-minute interval, the number of animals moving across the line and those moving into the circle were determined. The chemotaxis sensitivity was compared among different strains via percentage of animals moving into the circle:

$$\% \text{ animals present at the IAA spot} = \frac{\text{\# at the circle} \times 100}{\text{total \# of animals}}$$

Previous experiments on DEET and regular chemotaxis plates showed that the number of animals moving toward IAA reached a plateau and did not significantly increase thereafter. Therefore, the data collection was conducted at 15-minute intervals and was completed at 60 minutes. Although *C. elegans* tended to chemotax towards IAA, they did not remain at the same spot for very long. Consequently, prior to IAA addition, 1µl of 1M sodium azide was added at the circle to immobilize animals within a 0.5cm radius. Sodium azide is a solid and was diluted in H_2O. Hence, it did not interfere with the assay since IAA is a volatile attractant.

The **Chemotaxis assay** experimental protocol on well-fed adult animals was described by Bargmann and coworkers (1993) as well as by Troemel and colleagues (1997). Animals were placed in the middle of an agar plate. 1µl of an attractant and a neutral compound (such as ethanol) were placed at opposite ends of the plate. The number of animals at both spots were counted at 15-minute intervals for 60 minutes. Chemotaxis indexes were calculated as:

$$\text{Chemotaxis index} = \frac{\text{(number of animal at the attractant – number of animal at ethanol)}}{\text{total number of animals}}$$

Constructions of mutants

PH252 [*unc-44(e1197) egl-19(n582) IV*]- N2 wild-type males (+/+) were allowed to mate with MT1212 [*egl-19(n582) IV*] hermaphrodites. Two days after the cross was initiated, all N2 males were picked off the plate. Cross-progeny F1 males of this cross (*egl-19*/+) were then allowed to mate with CB1197 [*unc-44(e1197) IV*] hermaphrodites. Phenotypically wild-type L4 hermaphrodites (F2) were picked onto separate plates (4-5 animals/ plate) and were allowed to self-fertilize. Among the F3 hermaphrodites, the animals that expressed the Egl

phenotype were picked onto separate plates (4-5 animals/plate). F4 hermaphrodites were scanned for double mutants *egl-19(n582) unc-44(e1197)*. Due to the short distance between *egl-19(n582)* and *unc-44(e1197)*, approximate one thousand F3 Egl-19 animals were picked in order to find a double mutant.

PH253 [*unc-5(e152) lin-33(n1043) IV*]- the strategy to obtain this double mutant was similar to the one designed to make PH252 [*egl-19(n582)unc-44(e1197) IV*]. N2 wild-type males were allowed to mate with MT2121 [*lin-33(n1043) IV*] hermaphrodites. The male cross progeny (F1) were then allowed to mate with CB152 [*unc-5(e152) IV*] hermaphrodites. Phenotypically wild-type L4 hermaphrodites (F2) were chosen. Their progeny, F3 Lin-33 phenotype hermaphrodites, were picked and the F4 generation was examined for double mutants.

PH255 [*der-1(ky176) kyls156 (str-1:odr-10 CDNA-GFP int. on X)*], and PH256 [*der-1(ky176)kyls156 (str-1:odr-10 CDNA-GFP int. on X)*]-PH250 [*der-1(hf175) IV*] or PH251 [*der-1(hf176) IV*] male animals were crossed with CX5770 [*unc-44; kyls156 (str-1:odr-10 CDNA-GFP int. on X)*]. Phenotypically wild-type L4 hermaphrodites (F1) were picked onto separate plates (3-5 animals/ plate) and were allowed to self-fertilize. F2 animals were subjected to the DEET testing assay. Individual animals that moved toward the IAA spot were picked onto separate plates. DEET-resistant F3 animals were then examined for GFP expression.

3-Factor Crosses (3F Crosses)

As with other genetic models, 3F-Crosses constitute a powerful tool for genetic mapping in *C. elegans*. The rationale of this method was described in Wormbook (http://www.wormbook.org) and in Figure 1. The advantage of 3F Crosses over 2F Crosses is

the certainty of crossover location. This enables the investigator to focus on short distances that are often much harder to analyze, particularly when dealing with difficult phenotypes such as DEET resistance. In any 3F cross, two phenotypically distinguishable markers [in this case on a Linkage Group IV (LG)] were chosen and were present in a double mutant. This double mutant was crossed with male *der-1* animals. Among F2 progeny, rare recombinants, which are homozygous for only one marker, were picked and examined for the Der-1 phenotype after first making them homozygous for the recombinant chromosome.

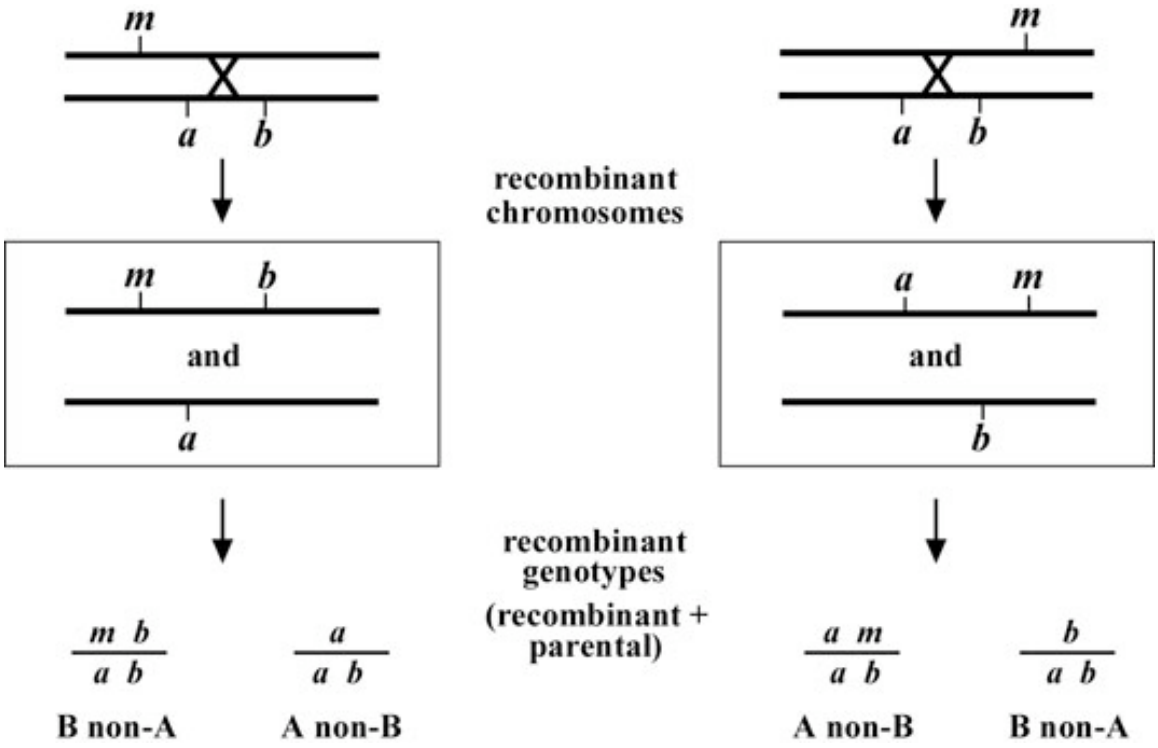

Figure 1. 3F cross scheme: F1 progeny was heterozygous in relation to mutant gene (m) and the two markers (a and b). Rare recombiants, which were homozygous for only one phenotype, were picked and allowed to self-fertilize. F3 animals were examined and were scanned for the presence of the mutant gene. If m was between a and b, then there would be a mixture of single mutant B non-A and double mutant (m and B, non-A) among the F2 B non-A animals. If m was to the left of a, then all B non-A exhibited the m phenotype. Conversely, if m was to the right of b, then all B non-A animals contained only B genotypes. (http://www.wormbook.org/chapters/www_threepointmapmarkers/threepo intmapmarkers.html).

In order to map *der-1*, either PH250 [*der-1(hf175) IV*] or PH251 [*der-1(hf176) IV*] males were crossed with a double mutant {either PH254 [*unc-5(e152) egl-19(n582) IV*],

PH252 [*egl-19(n582) unc-44(e1197) IV*], PH253 [*unc-5(e152) lin-33(n1043) IV*], PH135 [*unc-44(e1197) bli-6(sc16) IV*]}. Phenotypically wild-type L4 progeny hermaphrodites (F1) were picked onto separate plates (3-5 animals/plate) and were allowed to self-fertilize. Among the F2 animals, each non-Unc single mutant (*e.g.*, Egl-19 non-Unc-5, Egl-19 non-Unc-44, Lin-33 non-Unc-5, and Bli-6 non-Unc-44), which was the result of a recombination between the two markers, was picked on to a separate plate. Each recombinant was confirmed for homozygosity by the absence of Unc mutants in the F3 generation. Homozygous recombinants were subjected to DEET testing to determine the presence of the *der-1* mutation.

Complementation

A complementation test was used to determine whether the *der-1* mutations are alleles of *odr-8*. PH250 [*der-1(hf175) IV*] or PH251 [*der-1(hf176) IV*] males were crossed with CX2386 [*odr-8(ky31) IV*] hermaphrodites and CX2386 [*odr-8(ky31) IV*] males were crossed with either PH250 [*der-1(hf175) IV*] or PH251 [*der-1(hf176) IV*] hermaphrodites. The cross progeny were tested for the DEET-resistance phenotype using the DEET assay or for Odr-10-GFP localization using the Nomarski microscope.

Nomarski Microscopy

Animals were placed on thin agarose pads (5% agarose) on a microscope slide. GFP fluorescence was observed using the Nomarski Microscope Axioskop Fluorescence (Carl Zeiss, Inc).

Deficiency Mapping

Deficiency strains were crossed with either N2, PH250 [*der-1(hf175) IV*], or PH251 [*der-1(hf176) IV*] males. Male cross progeny were DEET-tested. Some deficiency strains expressed mutant phenotypes; therefore both male and hermaphrodite cross progeny were

counted in the DEET test. Animals from the cross plate were washed three times to dilute bacteria that might interfere with the DEET test. Animals, including self-progeny of the deficiency strain, cross progeny males and hermaphrodites, and Po males were placed on the DEET Assay plate but only the cross-progeny males, and/or hermaphrodites were counted throughout the test.

III. RESULTS:

3-Factor Crosses

For allele *hf176* (a.k.a Cand4A), out of 32 recombinants in the 3F Cross between *unc-5* (1.78mu) and *egl-19* (3.34mu), 19 were DEET resistant compared to 13 recombinants being sensitive to DEET. For allele *hf175*, half of the recombinants out of the total of 22 were DEET-resistant. The results put the mapping distance of *hf176* and *hf175* on the same location on Linkage Group IV. This strongly suggests that *hf175* and *hf176* are allelic and that *der-1* is located between *unc-5* and *egl-19*. This conclusion was further supported by another 3F cross between *unc-5* and *lin-33* (2.55mu). All the Lin-33 non-Unc-5 recombinants, generated from both *hf176* and *hf175*, were DEET sensitive (Figure 2).

However, the one additional 3F cross that was conducted with the hope to further solidify the map position of *der-1* contradicted the two former crosses. Specifically, the data between *unc-44* (2.92mu) and *egl-19* (3.34mu) suggested that the map location of *hf176* is to the right of *egl-19*. Conversely, the test results suggest that *hf175* is located between *egl-19* and *unc-44*. Thus, these results do not support the notion that *hf175* and *hf176* are allelic. In addition, they are mutually exclusive from the 3F-cross results presented in the previous paragraph.

RNAi

There are three methods of delivering dsRNA to *C. elegans* (Ahringer, 2006). The most convenient of these is to supply animals with *E. coli* that produce dsRNA. The RNAi experiments described in this section employed this, the feeding method. The premise was that RNAi would knock down putative gene expression in wild-type animals and a single RNAi strain corresponding to *der-1* would render their progeny DEET resistant. A complete list of all RNAi candidates tested is showed in Table 1. Unfortunately, data from many RNAi

tests showed a high background and were negative in the sense that there was not a single gene whose transcriptional knockdown appreciably altered the response to DEET (Figure 3). These experiments were conducted on at least three different occasions with essentially the same results each time. The result did not come as a surprise because RNAi is known to be less effective in neurons (Jansen, 2002), which are the presumed target of DEET action.

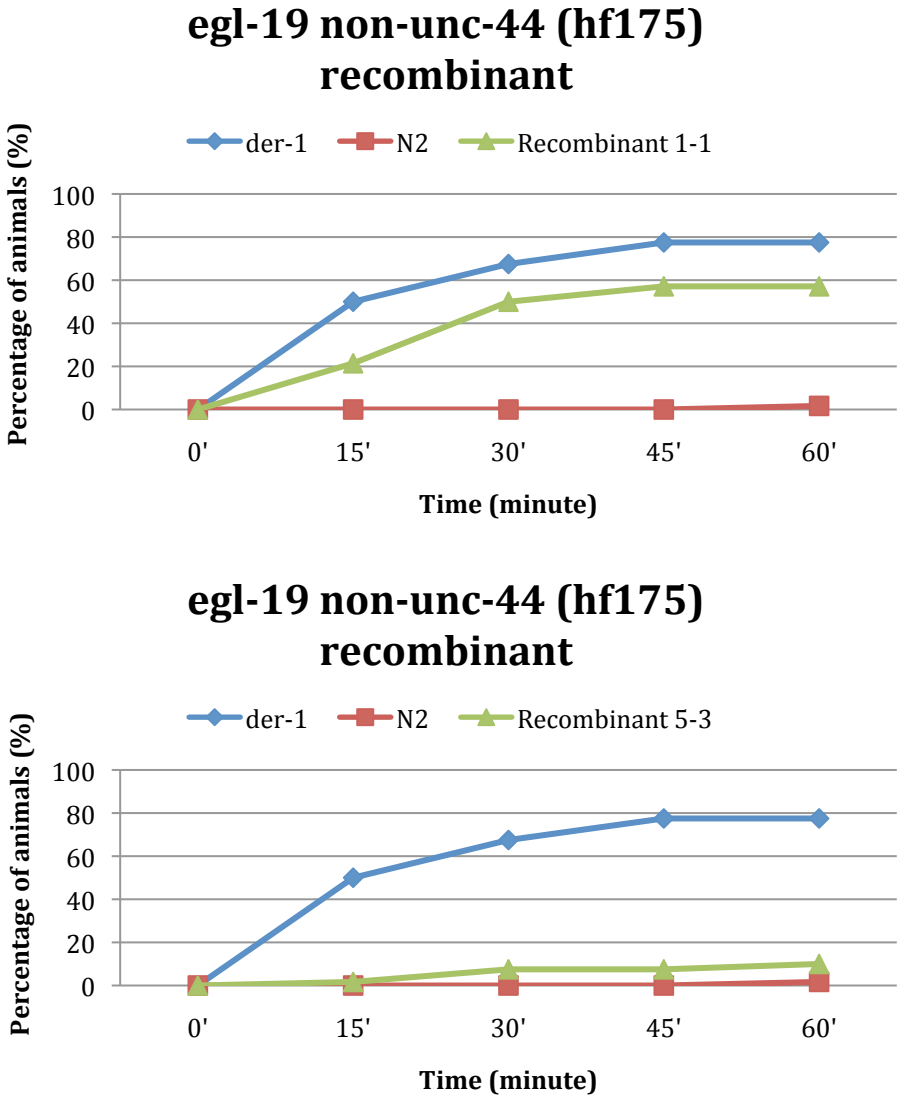

Figure 2. Each recombinant was evaluated by performing a DEET Testing Assay. Data were graphed as the percentage of animals chemotaxing toward the IAA spot every 15 minutes for a total of 60 minutes. The DEET-resistant behavior of each recombinant was assessed based on their similarity with either the negative control (N2) or the positive control (*hf175* or *hf176*). In this case, 1-1 is a recombinant that was ultimately scored as DEET sensitive while is a recombinant that was DEET resistant.

Genes	Protein	Position (mu)	Position (bp)
C39H7.5*	Srsx-14	IV: 1.94	IV: 5,627,141..5,624,840
C39H7.6*	Srd-12	IV:1.94	IV: 5,630,224..5,628,777
B0547.4*	Srd-8	IV: 1.95	IV: 5,645,599..5,643,237
T19E7.5*	Srd-5	IV: 2.35	IV: 5,674,527..5,672,618
C55C3.4*	C55C3.4	IV: 2.35	IV: 5,678,129..5,676,197
C55C3.1*	C55C3.1	IV: 2.37	IV: 5,709,081..5,714,321
C55C3.2*	C55C3.2	IV: 2.37	IV: 5,707,081..5,708,402
C45E5.4*	C45E5.4	IV: 2.38	IV: 5,735,004..5,737,089
C31H1.7*	C31H1.7	IV: 2.42	IV: 5,810,288..5,808,565
C06E7.2*	C06E7.2	IV: 2.44	IV: 5,839,384..5,843,980
C06E7.3*	Sams-4	IV: 2.44	IV: 5,846,383..5,843,967
C06E7.7*	Srv-17	IV: 2.46	IV: 5,870,157..5,867,674
H04M03.6*	Srv-19	IV: 2.46	IV: 5,874,066..5,872,050
H04M03.11*	H04M03.11	IV: 2.48	IV: 5,883,876..5,882,737
H04M03.2*	Nspb-6	IV: 2.50	IV: 5,895,480..5,895,839
H32C10.3*	H32C10.3	IV: 2.53	IV: 5,916,004..5,921,380
H32C10.2*	Lin-33	IV: 2.55	IV: 5,930,629..5,933,249
H32C10.1*	H32C10.1	IV: 2.53	IV: 5,934,428..5,937,429
K02B2.4*	Inx-7	IV: 2.64	IV: 5,946,663..5,943,965
T22D1.3	T22D1.3 (IV-3K12)	IV: 3.25	IV: 6,913,518..6,917,094
T22D1.2	T22D1.2 (IV-3K10)	IV: 3.25	IV: 6,921,839..6,922,836
B0478.2	Transposon (IV-3M10)		IV: 6,947,709..6,950,421
C25A8.2	C25A8.2 (IV-3006)	IV: 3.27	IV: 7,009,872..7,010,728
C06G3.8	C06G3.8 (IV-3B01)	IV: 3.28	IV: 7,031,630..7,032,543
C06G3.9	C06G3.9 (IV-3B03)	IV: 3.28	IV: 7,032,826..7,036,843
C06G3.3	C06G3.3 (IV-3016)	IV: 3.28	IV: 7,036,113..7,036,362

Table 1. Possible genes were tested for DEET-resistance via feeding adult animals with appropriate RNAi strains. F1 adults were subjected to DEET assays(*: Tests were performed by Case, C., 2008). These experiments were done concurrently with 3-Factor Cross experiments; therefore, various candidates from a broad region were tested. As more 3F-Cross data were available, the search was focused between 2.4- 2.9mu.

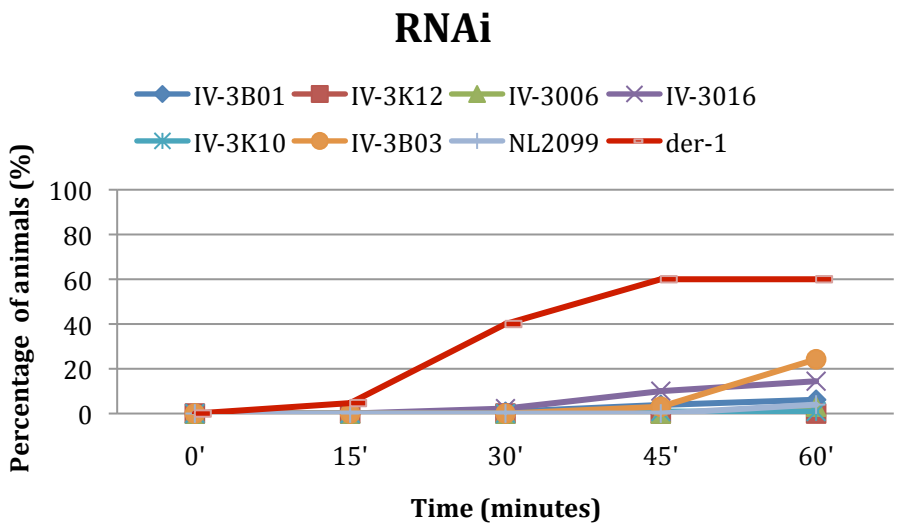

Figure 3. The percentage of animals that accumulated in the IAA spot were counted every 15 minutes for total of 60 minutes. All animals from different RNAi plates showed similar behaviors towards the attractant in the presence of DEET (including IV-3006, IV-3B01, IV-3B16, and IV-3M10, data not shown). The data suggest that none of these genes are the same as *der-1*.

Mutant candidates

Genes	Allele	Strain	Position (mu)	Position (bp)
Srv-17	ttTi27648	-	2.46	5,870,157..5,867,674
Srv-22	ttTi7473	-	2.47	5,879,938..5,878,455
T22D1.12	tm1498	-	3.25	6,931,102..6,929,072
Nhr-249*	tm3367	-	3.25	6,972,511..6,971,882
T22D1.11	ok2338	RB1804	3.25	6,928,404..6,926,150
Jnk-1	gk7	VC8	3.25	6,957,625..6,963,709
C06G3.7	ok2580	RB1961	3.28	7,011,987..7,016,526
C06G3.6*	tm0418	-	3.28	7,017,610..7,020,186
Srg-50*	tm2701	-	3.29	7,075,147..7,074,014
Srg-50*	tm2717	-	3.29	7,075,147..7,074,014

Table 2. Lists of other candidate strains that were tested for DEET resistant phenotype.(*: Courtesy of Dr. Mitani, S., Tokyo Women's Medical University School of Medicine, Tokyo, Japan)

The *odr-8* gene was mapped in the Bargmann laboratory (personal communication) to 2.67mu, which overlaps the region where the *der-1* gene is located based on the 3F cross mapping data (see above). To test whether the mutations that define *odr-8* (isolated in Bargmann's laboratory on the basis of a chemotaxis defect towards benzaldehyde) and *der-1*

(isolated in our lab on the basis of DEET resistance) are allelic, DEET assays were performed on all *odr-8* alleles. Despite some initial indications to the contrary, the DEET assay results eventually showed strong sensitive responses of *odr-8* towards IAA and benzaldehyde (data not shown). In addition, Der-1 animals were strongly attracted to benzaldehyde (even in the presence of DEET), which is a marked departure from the behavior of *odr-8* mutants. Finally, unlike *odr-8; odr-10::GFP* (Dywer et al., 1998), *odr-8/der-1, and odr-10::GFP* animals had normal transmembrane protein Odr-10 localization to the AWA cilia (data not shown). Collectively these data indicate that *odr-8* is not the same gene as *der-1*. Besides *odr-8*, other candidate strains that were tested for the DEET-resistant phenotype are listed in Table 2. None of these candidates proved to be DEET resistant.

Deficiency Mapping

The premise of deficiency mapping is that if a segment of chromosome that contains a gene of interest (in this case *der-1*) is omitted, the phenotype of the *der-1*/*Deficiency* heterozygote will be mutant (in this case DEET resistant). Out of nine deficiencies over the 1.7 – 3.2mu area, none was found to exhibit DEET resistance (Figure 4). The data, however, does not mean that *der-1* was not within the region spanned by one or more of the deficiencies. Since male animals were tested in these experiments and that male animals have several additional classes of chemosensory neurons that contribute to mating (Liu and Sternberg, 1995, Sulston et al., 1980), these may also contribute to DEET sensitivity.

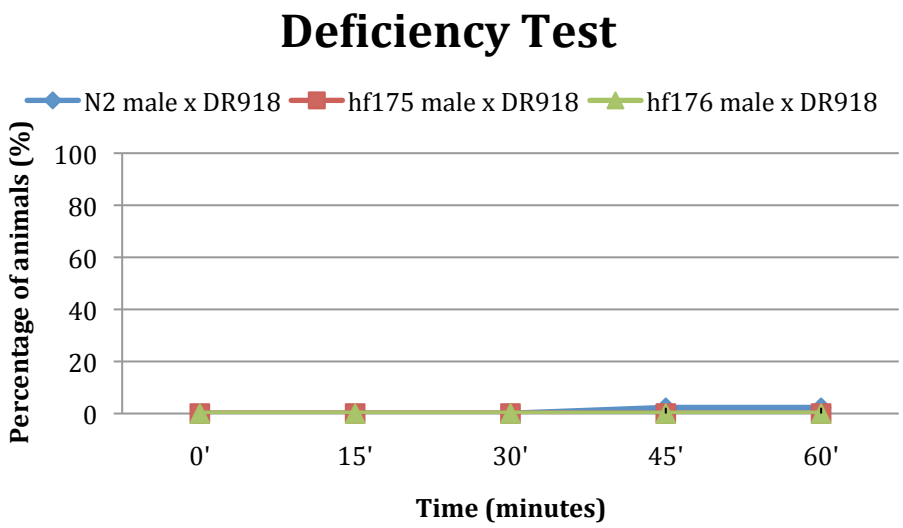

Figure 4. Deficiency mapping. The percentage of male cross-progeny was graphed as a function of 15' increments for 60 minutes. Nine deficiency strains were tested: CB3824, DR918, DR814, BC1216, RW1350, DA768, DR799, BC1217, and DR684. All nine tests proved to be negative.

Other wild-type strains of *C. elegans*

In the course of performing various experiments, it was discovered that the DEET-sensitive phenotype was not universal among other wild-type strains of *C. elegans*. Out of five wild-type strains available in the lab, three of them (CB4856, AB1, and AB2) proved to be DEET resistant as opposed to DEET-sensitive (N2 and CB4852). Intriguingly, two of the three DEET-resistant wild-type strains were located in Australia and the third was isolated in Hawaii. Geographically, these strains are indigenous to the Pacific islands. Conversely, N2 and CB4852 were isolated in Britain, which is geographically disparate from the Pacific islands.

IV. DISCUSSION:

3F Cross data suggested that *der-1* (both alleles *hf175* and *hf176*) is located very close to *lin-33* (2.55mu). It is approximately 200kbps to the left and to the right of *lin-33*. We hypothesize that *der-1* encodes a chemosensory receptor based on the composite list of putative genes within 1.78mu and 3.39mu from *C. elegans* database (Wormbase). *C. elegans*, like other animals, has G-protein-coupled receptors (GPCRs) with seven transmembrane domains. It has been suggested that *C. elegans* has approximately 1300 predicted genes that encode members of putative chemosensory receptors (Troemel, Kimmel and Bargmann, 1995). These are grouped in different superfamilies depending on their compositions and functions. Moreover, there are an additional 400 chemosensory receptor pseudogenes (Troemel, Kimmel and Bargmann, 1995). In some cases, the activity statuses of these pseudogenes were not the same among different wild-type strains. For example, it was shown that some of the inactive pseudogenes of two families *srh* and *str* in N2 are actually active in other strains (Stewart, 2004). This may bear on the question of why some wild-type strains are DEET resistant while others are DEET sensitive (Figure 5). The classical genetics definition of wild type is "that type found in the wild." The assumption is that the wild type has functional alleles of various genes and that mutants isolated from the wild type are defective for that particular function. In the case of DEET resistance, the assumption was that the wild-type *der-1* allele in N2 is functional and confers DEET sensitivity. Further, it was assumed that the EMS mutagenesis of N2 resulted in a null or hypomorphic allele that rendered animals DEET resistant. However, the presence of other wild-type strains that are DEET resistant calls this into question. It is at least plausible that "wild-type" N2 possesses a non-functional allele that confers DEET sensitivity. If so, EMS mutagenesis could have resulted in either an intragenic or extragenic suppression of DEET sensitivity. In this regard,

Steward and colleagues (2004) also showed that in more than half (~50) of these pseudogenes in the *srh* and *str* superfamilies, there was one common defect: a stop codon or a deletion, either of which can be created with EMS. The common type of mutation induced by EMS is G/C-A/T transitions, although it does cause some small deletions and chromosomal rearrangements (Anderson, 1995). Scanning through potential candidate genes among all known serpentine receptors, pseudogene *srd-6* was mapped to 1.95mu, which is closed to where *der-1* was mapped. If *der-1* is *srd-6* and somehow a mutation on *srd-6* reverted the nonfunctional protein to its normal function, this could be used to explain the DEET-resistant characteristics in other wild-type strains of *C. elegans*. Another possibility would be the higher probability of misalignment of chromosome IV during meiosis or replication due to the cluster of four serpentine receptor proteins, *srv*, located at 2.3 – 2.5mu. Either unequal crossing over or replication slippage could convert active genes into pseudogenes (and visa versa).

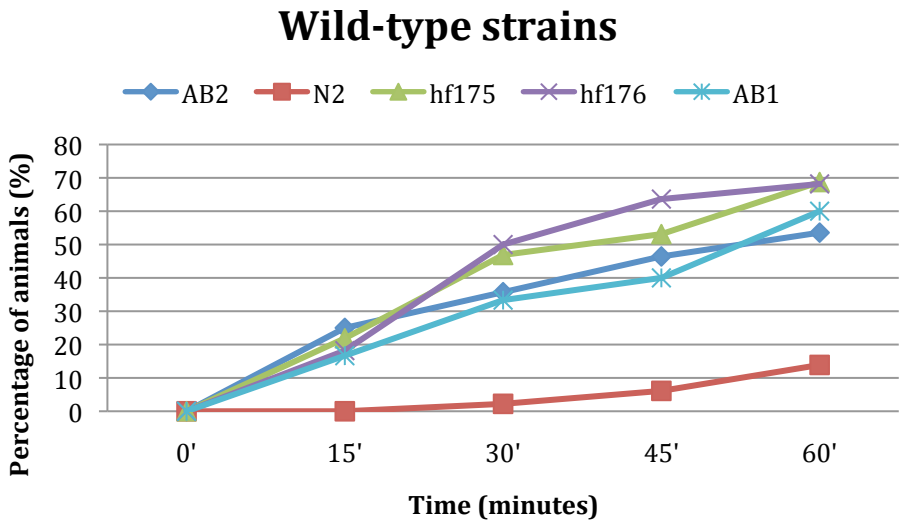

Figure 5. Percentage of animal at the attractant spot (y-axis) vs. time (minutes). AB1 and AB2 were as DEET-resistant as Der-1 compared to the DEET-sensitive N2 strain.

Although DEET interfered with *C. elegans* chemotaxis towards IAA, we observed that the effect seemed to attenuate over time. Approximately three hours after the animals were put onto a DEET plate, the majority of DEET-sensitive and DEET-resistant cumulated at the attractant spot (unpublished data). This suggested that either *C. elegans* adapted to DEET (behavioral plasticity) or there was some level of receptor saturation.

In addition, iso-amyl alcohol was the only attractant employed in this study. Since the mode of behavior in *C. elegans* varies among attractants or repellents, it is important to investigate the DEET-sensitive and DEET-resistant behaviors with respect to other volatile substance as well as non-volatile chemicals.

To further investigate the effect of DEET on the chemotaxis of *der-1* towards volatile substances, the animals were tested for their behaviors towards repellents in the presence of DEET (Bargmann et al., 1993). We have some preliminary data showing that DEET interfered with *C. elegans* ability to sense repellents. Specifically, the repellent behavior produced by *der-1* and N2 was significantly less profound compared to their behaviors in a DEET-free environment (Table 3). If DEET acted as a universal inhibitor of olfaction, then we expected the chemotaxis index of DEET-sensitive strains towards 2-octanol, a repellent, to be close to 0 and of *der-1* to be negative. Conversely, the results strengthen the argument that *der-1* does not inhibit olfaction universally and instead may have specific and different affects dependent upon the specific chemical (attractant versus repellent; volatile versus non-volatile).

	Wild-type N2	Hf175	Hf176
Without DEET	-0.202 $\pm$ 0.120	0.225 $\pm$ 0.115	-0.49 $\pm$ 0.078
With DEET	-0.038 $\pm$ 0.047	-0.035 $\pm$ 0.050	-0.090$\pm$ 0.059

Table 3. Chemotaxis test of Der-1 on 2-octanol, which is a repellent, showed that DEET interfered with the animals' response. The difference was profound when DEET was present. It was not, however, among wild-type N2 and Der-1.

DEET-resistance varies among wild-type strains that were geographically isolated. Why wild-type strains would be DEET-resistant is not known. The phenotype could have originated from some pleiotropic genes or could be due to some unknown evolutionary pressure. An attempt to look at the geography of DEET resistance is under way.

Microinjection (a.k.a. cosmid/fosmid rescue) is a useful procedure to clone a mutant gene (Evans, 2006). In this process, adult animals are injected with the normal copy of dsDNA into the distal arm of the gonad, which contains a central core of cytoplasm that is shared by many germ cell nuclei. Consequently, the injected DNA can be incorporated in many progeny. The appropriate piece of DNA (a.k.a cosmid/fosmid) will "rescue" the mutant from expressing the mutant phenotype (in this case DEET resistance). Nevertheless, microinjection usually works well with loss-of-function mutations rather than gain-of function ones. If the *der-1* mutations were gain-of-function, then the rescue experiment may not bring about a positive result. One alternative would be to narrow down possible candidate genes and sequence the coding regions, comparing N2, *der-1(hf175)*, and *der-1(hf165)* to look for mutations. In fact, since there are only a handful of gene candidates in the region most likely to encompass *der-1*, this candidate gene approach is likely the most feasible.

In the past 50 years, global warming has broadened the active zone of many infectious and deadly bacteria and insects (Climate Institute). Given that, the demand for a more effective insect repellent has become a potential elixir in preventative medicine. Successful cloning of *der-1* may elucidate the mechanism of DEET action and may potentially help with designing the next generation of insect repellent products that reduce insect-borne diseases such as malaria, a prominent problem in third-world countries, especially Africa.

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ABSTRACT

THE GENETICS OF DEET-RESISTANCE IN *CAENORHABDITIS ELEGANS*

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The molecular mechanism(s) by which DEET (N,N-diethyl-m-toluamide) acts as an insect "repellent" has not been resolved. Using forward genetic screens in *Caenorhabditis elegans*, we have isolated five DEET-resistant mutants *(der-1)* after EMS mutagenesis of DEET-sensitive N2. Two are allelic and have been mapped between 2.4 mu and 2.9 mu on linkage group (LG) IV. Specifically, 3F (factor) cross data between two markers *unc-5* (1.78 mu) and *egl-19* (3.34 mu) on LGIV showed that 11/22 (50%) Egl-19 recombinants were DEET-resistant and 11/22 (50%) were DEET sensitive for allele *hf175*. In case of allele *hf176*, the data were 19/32 (59%) and 13/32 (41%), respectively. In addition, all Lin-33 recombinants from the 3F cross between two references *unc-5* (1.78 mu) and *lin-33* (2.55 mu) showed DEET sensitivity in both *hf175* and *hf176*. Several additional 3F crosses are in various stages of completion. Interestingly, wild-type strains CB4856, AB1, and AB2 have been shown to be DEET resistant relative to the wild-type strains N2 and CB4852, which are both DEET sensitive. These findings suggest that DEET-resistance may be more common than we originally expected. Successful cloning of the *der-1* gene is an important step not only to elucidate the mechanism of DEET action but may prove useful in designing the next generation of chemicals that helps reducing insect-borne-diseases.